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REVIEW ARTICLE

Genomic Changes Defining the Genesis, Progression, and Malignancy Potential in Solid Human Tumors: A Phenotype/Genotype Correlation

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The transition of normal epithelium to invasive carcinoma occurs sequentially. In colorectal and cervical carcinogenesis, this transition is reflected by histomorphologically defined grades of increasing dysplasia that untreated may progress to invasive disease. In an attempt to understand the role of chromosomal aberrations during tumorigenesis we have applied comparative genomic hybridization using DNA extracted from defined stages of colorectal and cervical tumors, from low- and high-grade astrocytic tumors and from diploid and aneuploid breast carcinomas. Genetic instability, as measured by the number of chromosomal copy alterations per case, increases significantly at the transition from precursor lesions to invasive carcinomas and continues to increase with tumor stage. Aggressive tumors have a higher number of copy alterations per case. High-level copy number changes (amplifications) become more prevalent in advanced-stage disease. Subtractive karyograms of chromosomal gains and losses were used to map tumor stage-specific chromosomal aberrations and clearly showed that nonrandom chromosomal aberrations occur during disease progression. In colorectal and cervical tumors, chromosomal copy number changes were correlated with nuclear DNA content, proliferative activity, expression levels of the tumor suppressor gene *TP53*, and the cyclin-dependent kinase inhibitor *p21/WAF1*, as well as the presence of viral genomes. Here we summarize and review the results of this comprehensive phenotype/genotype correlation and discuss the relevance of stage-specific chromosomal aberrations with respect to diagnostic applications. *Genes Chromosomes Cancer 25:195–204, 1999.*

INTRODUCTION

The sequential transformation of normal epithelium to invasive carcinoma is reflected by increasing levels of cellular and histomorphological alterations. The possibility to detect both colorectal and cervical tumors at early stages of disease progression makes these tumors valued model systems to study progressive genetic changes and their phenotypic consequences in clinical samples. In particular, in colorectal tumors the dissection of molecular events in invasive carcinomas and their precursor lesions has greatly contributed to our understanding of the multistep nature of carcinogenesis (Fearon and Vogelstein, 1990). In cancer cells, the functional gain or loss of oncogenes and tumor suppressor genes, respectively, is often mirrored by the acquisition of chromosomal aberrations. Therefore, gene copy number changes required for tumor initiation and progression can be detected cytogenetically as chromosomal deletions, monosomies, duplications, polysomies, and as correlates of gene amplifications, such as homogeneously staining regions (hsr) or double minute chromosomes (dmin) (Heim and Mitelman, 1995).

The introduction of comparative genomic hybridization (CGH) as a molecular cytogenetic screening test for copy number changes in tumor genomes has expanded the spectrum of methodologies that can be used in addition to existing techniques, such as chromosome banding analyses, to identify recurring chromosomal aberrations (Kallioniemi et al., 1992, 1993; du Manoir et al., 1993). CGH has been used to screen virtually all common human cancers and has revealed in all instances a tumor-specific blueprint of chromosomal copy number changes (Forozan et al., 1997; Ried et al., 1997; Zitzelsberger et al., 1997). In a CGH experiment, only genomic tumor DNA is required for analysis. Therefore, previously fixed and paraffin-embedded tissue sections can be used for this comprehensive cytogenetic screening test as well (Speicher et al., 1993; Isola et al., 1994). This makes CGH an ideal tool for the analysis of copy number changes in histologically defined stages of solid tumor progression and, retrospectively, in tumors from patients with re-

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ported clinical follow-up. The possibility to use DNA extracted from histologically defined tissues also permits the serial analysis of additional pertinent parameters, such as DNA ploidy, the expression of tumor-relevant genes, and the detection of viral genomes on consecutive sections from identical tumor samples.

In this review, we summarize the results of recent studies where we have applied CGH to screen for chromosomal copy number changes in progressive stages of colorectal and cervical carcinogenesis (Heselmeyer et al., 1996, 1997b; Ried et al., 1996), in low- and high-grade astrocytic tumors (Schröck et al., 1994, 1996), in diploid and aneuploid breast carcinomas (Ried et al., 1995), in lung carcinomas (Ried et al., 1994; Petersen et al., 1997), and in carcinomas of the fallopian tube (Heselmeyer et al., 1998). This summary focuses on the role of increased genetic instability as measured by the average number of copy alterations (ANCA) during tumor progression, on the cellular phenomena that allow the fixation of specific chromosomal changes, and on the identification of chromosomal aberrations that occur at certain tumor stages by means of subtractive karyograms of DNA gains and losses. Finally, we discuss the applicability of these chromosomal markers of tumor progression to routine diagnosis and staging of premalignant lesions and invasive carcinomas.

RESULTS AND DISCUSSION

Average Number of Copy Alterations

The average number of copy alterations (ANCA) index is a measure for the number of acquired chromosomal copy alterations in a given tumor type or at specific stages of a given tumor type. It is deduced by dividing the total number of copy alterations presented in a karyogram of gains and losses by the number of tumors analyzed. For instance, 110 chromosomal aberrations were mapped in a series of 23 squamous cell carcinomas of the anal canal, resulting in an ANCA of 4.8 (Heselmeyer et al., 1997a). These tumors have in general a favorable prognosis. In colorectal and cervical carcinogenesis, ANCA clearly increases with increasing cellular atypia. The sequence presents as follows: low-grade colorectal adenomas, 0.2; high-grade adenomas, 1.9; and invasive carcinomas, 5.6 (Ried et al., 1996). A similar picture emerges during cervical carcinogenesis: moderate and severe dysplasia have an ANCA of 0.4; invasive carcinomas stage I, 4.0; and invasive carcinomas, stage IIb–IV, 8.2 (Heselmeyer et al., 1996, 1997b). Diploid and aneuploid breast carcinomas that follow a strikingly different clinical course (Auer et al., 1980, 1984; Fallenius et al., 1988) also differ significantly in their ANCA values: benign fibroadenomas show no chromosomal aberrations (ANCA of 0), carcinomas with favorable prognosis reveal an average of 2.4 aberrations, and the ANCA in aggressive, aneuploid tumors is 6.8 (Ried et al., 1995). In a series of 10 astrocytic tumors (grade II) in adult patients, the ANCA index is 2.1 (Schröck et al., 1996), whereas in glioblastoma multiforme (astrocytic tumors grade IV), the ANCA increases to 9 (Schröck et al., 1994). In small-cell lung carcinomas removed during autopsy, the ANCA is 13, meaning that each tumor analyzed has acquired, on average, 13 chromosomal aberrations (Ried et al., 1994; Petersen et al., 1997). The ANCA index in primary carcinomas of the fallopian tube, which is an exceedingly aggressive tumor, even exceeds the ANCA in small-cell lung cancers and amounts to 19.7 (Heselmeyer et al., 1998). The data are summarized in Figure 1.

In addition to the identification of chromosomal gains and losses, CGH also permits the chromosomal mapping of high-level copy number changes, such as whole arm and chromosome band-specific amplification events. Such high-level copy number increases or amplifications become more prevalent with advanced tumor stage during both colorectal and cervical carcinogenesis. Furthermore, the ratio of amplification events compared to low-level copy number changes (such as trisomies or monosomies) increases as well. In premalignant lesions, high-level copy number gains are rare and are present only in 0.08 per case in the collection of 26 premalignant colorectal adenomas (4.1% of all chromosomal copy number changes). None of the 23 cervical intraepithelial neoplasias revealed gene amplifications. However, in invasive carcinomas, amplification events become common. In colorectal carcinomas, 1.2 amplifications were identified per case, which amounts to 20% of all copy number changes identified in invasive colon carcinomas. The incidence in stage I invasive cervical carcinomas is 7% of all aberrations, which increases to 9% in advanced-stage disease. In diploid breast adenocarcinomas, 8.3% of the aberrations could be identified as high-level copy number increases, whereas amplifications contribute to 19% of all aberrations in aneuploid carcinomas (Ried et al., 1995). In low-grade astrocytic tumors and glioblastoma multiforme, the numbers are 9% and 13%, respectively (Schröck et al., 1994, 1996). In small-cell lung cancers, 18% of the aberrations were mapped as amplification events (Ried et al., 1994). The num-

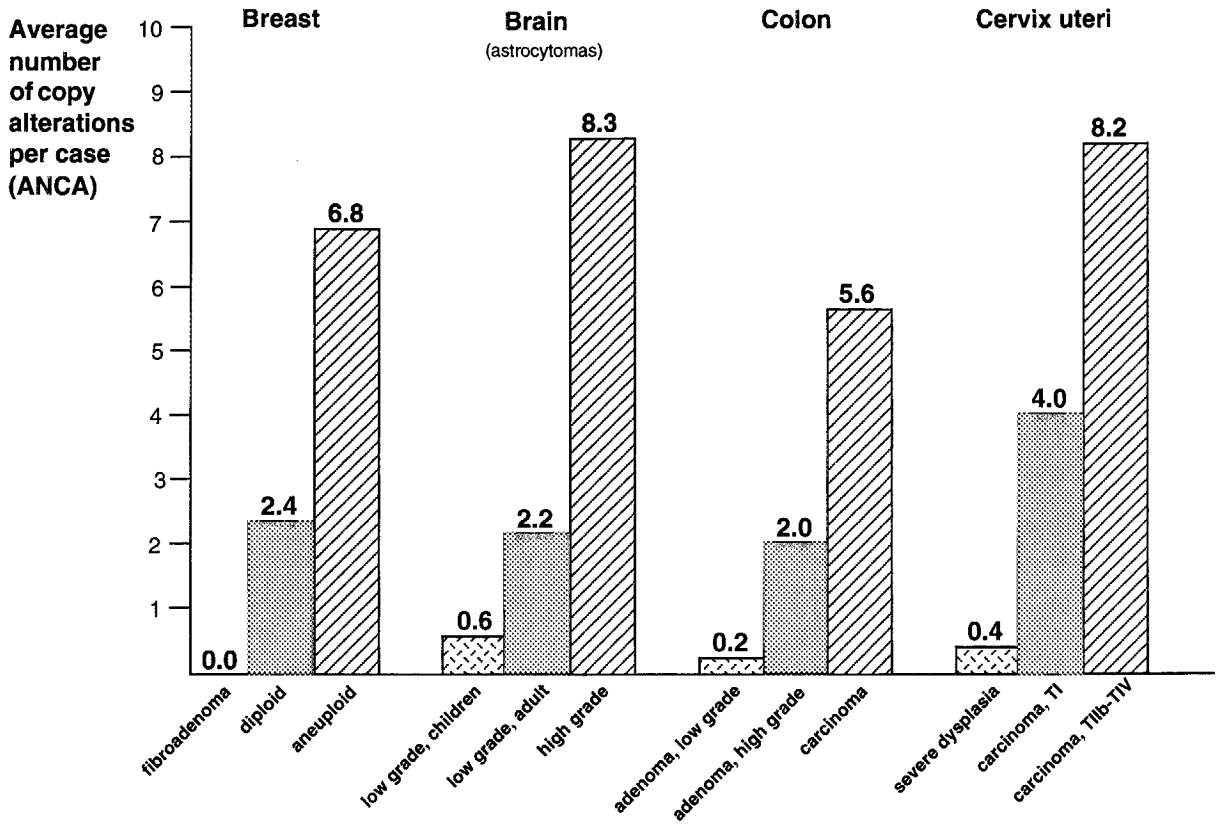

Figure 1. Average number of copy alterations (ANCA) detected by CGH. The figure presents the ANCA detected in breast tumors (fibroadenomas and diploid and aneuploid adenocarcinomas), brain tumors (low-grade astrocytic tumors in children and adults and glioblastomas multiforme), colorectal tumors (low- and high-grade adenomas and invasive carcinomas), and tumors of the uterine cervix (severe dysplasia, stage I, and advanced-stage invasive carcinomas). The ANCA index is computed by dividing the total number of chromosomal aberrations detected by CGH by the number of samples analyzed. The results are based on the following CGH-studies: Schröck et al. (1994, 1996), Ried et al. (1995, 1996), and Heselmeyer et al. (1996, 1997b).

ber of amplification events and the percentage of amplification events with respect to the total number of copy alterations is presented in Figure 2 for breast, brain, colorectal, and cervical tumors. It is noteworthy in this respect that high-level copy number changes are relatively infrequent at early stages. This could be due to the fact that the copy number increase of many genes located on, e.g., chromosome arm 3q is beneficial to render an initial growth advantage to the cell. More localized high-level copy number changes are important at later stages as a response to selective environmental pressure. Alternatively, the gain or loss of whole chromosomes or chromosomal arms can occur without a pronounced compromise of genetic stability, and this maintenance of cellular control mechanisms at early stages of carcinogenesis would allow the cells to acquire whole chromosome or chromosomal arm aberrations more easily than regional copy number changes such as gene amplifications. The acquisition of high-level changes, however, seems to require severely compromised cell cycle control, for instance via the impairment or elimination of *TP53* function.

In summary, the number of chromosomal aberrations clearly correlates with disease progression and seems to be a valid marker for increased genomic instability. The findings in colorectal and cervical carcinogenesis are in line with results obtained in other tumors. The results show that ANCA correlates with disease progression and with prognosis. Therefore, the ANCA index might be a valid parameter for the assessment of tumor malignancy.

Identification of Tumor Stage-Specific Chromosomal Aberrations by Means of Subtractive Karyograms of Gains and Losses

The clear correlation of the ANCA index with tumor progression or tumor aggressiveness is a reflection of increasingly unstable genomes. However, it would be exceedingly interesting to analyze if such copy number alterations are a mere reflection of advanced cellular dysplasia and progressing tumor stages or if they occur as a sequence of

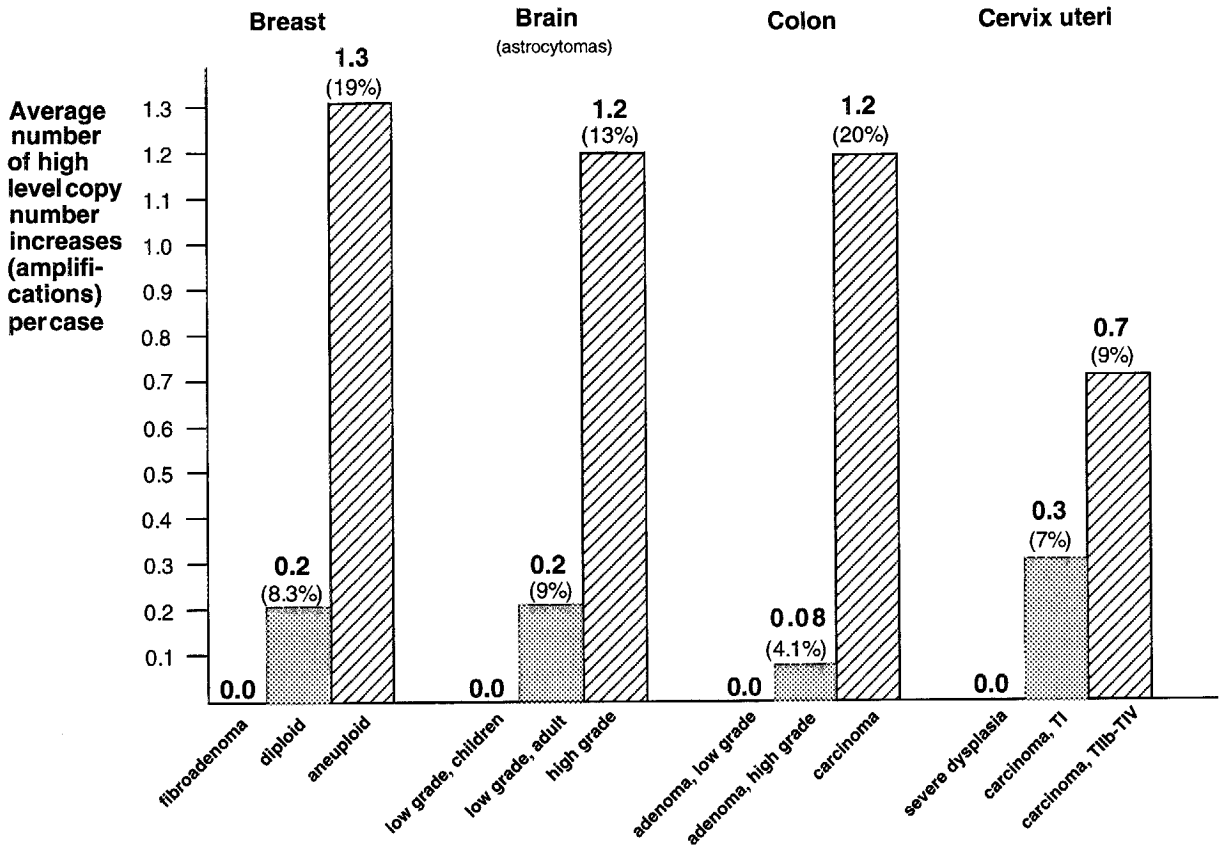

Figure 2. High-level copy number increases (amplifications) as a function of tumor progression. This figure shows the number of high-level copy number increases (amplifications) in breast tumors (fibroadenomas and diploid and aneuploid adenocarcinomas), brain tumors (low-grade astrocytic tumors in children and adults and glioblastomas multiforme), colorectal tumors (low- and high-grade adenomas and invasive carcinomas), and tumors of the uterine cervix (severe dysplasia, stage I, and advanced-stage invasive carcinomas). See also Figure 1 for comparison. The percentage of amplifications vs. low-level copy number changes such as monosomies or trisomies is presented in parentheses. The average number of high-level copy number increases (amplifications) is computed by dividing the number of amplification events by the number of cases analyzed. The number as well as the ratio of amplification events clearly increases with advanced disease stages. The results are based on the following CGH-studies: Schröck et al. (1994, 1996), Ried et al. (1995, 1996), and Heselmeyer et al. (1996, 1997b).

chromosomal aberrations that reflect a pattern of stage-specific chromosomal changes. In other words, does CGH allow one to map specific chromosomal aberrations during the genesis of solid tumors? In order to address this question of sequential yet specific chromosomal changes, we have introduced subtractive karyograms of chromosomal gains and losses. For instance, aberrations that were already detected in high-grade colorectal adenomas were subtracted from those present in invasive carcinomas, and those detected in stage I invasive carcinomas from aberrations mapped in advanced-stage disease. The subtractive karyograms equal the results expected from a CGH experiment that would utilize DNA extracted from advanced-stage disease as the test DNA and DNA extracted from low-stage disease as the control genome. Subtractive karyograms are displayed for frequently involved chromosomes both in colorectal and cervical carcinogenesis. Figure 3 exemplifies the results. In colorectal carcinomas, chromosome arms 7p (gained in 50% of the cases), 8q (56%), 13q (56%), and 20q (75%) are commonly gained, while chromosome arm 8p (45%) is frequently lost. However, a gain of both chromosome arm 7p and chromosome 20 is already present in higher copy numbers in 33% of high-grade colorectal adenomas. Chromosomes 8 and 13, on the other hand, were gained at much lower frequencies (16% and 8%, respectively), and no loss of chromosome arm 8p was observed. We therefore conclude that copy number increases of chromosomes 7 and 20 are early events in colorectal carcinogenesis and precede the acquisition of copy number increases on chromosome arms 8q and 13q and the loss of chromosome arm 8p. Thus, these aberrations occur rather late in tumor development, i.e., at the transition of high-grade adenomas to invasive disease.

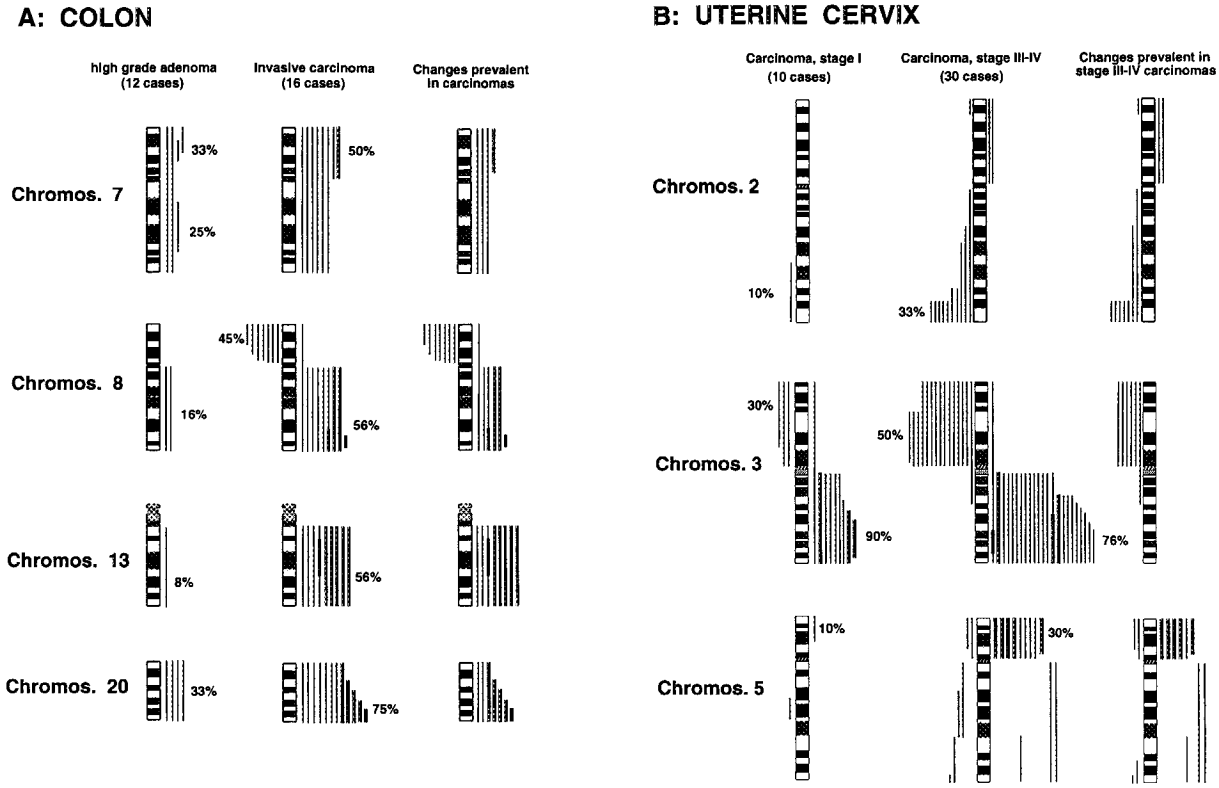

Figure 3. Subtractive karyograms of chromosomal gains and losses in colorectal and cervical carcinogenesis. The subtractive karyograms of gains and losses were generated to discern early from late chromosomal aberrations in the progression of colorectal and cervical tumors. These karyograms are generated by subtracting copy number changes in early lesions from those prevalent in advanced-stage disease. The percentages indicate the frequency of these aberrations, taking into account the different case numbers that are provided in parentheses with the histological classification. Lines of the right side of the chromosome ideograms reflect DNA gains, lines of the left DNA losses. Bold lines indicate high-level copy number increases. The case numbers are indicated below the subtitles. The subtractive karyograms are based on CGH analyses by Heselmeyer et al. (1996; 1997b) and Ried et al. (1996).

A: In colorectal carcinomas the gain of sequences on chromosome arm 7p was detected in 33% of high-grade adenomas and increased slightly to 50% in invasive carcinomas. Loss of chromosome arm 8p and gain of chromosome 13, however, were mapped with much lower frequency in high-grade adenomas compared to invasive carcinomas. Gain of chromosome 7 was therefore interpreted to occur early in tumor progression, whereas the loss of chromosome arm 8p and gain of chromosome 13 occur at later stages. **B:** In cervical carcinomas, the gain of chromosome arm 3q occurs in 90% of stage I disease, but at slightly reduced frequency in advanced-stage carcinomas. All other aberrations occur with higher frequency in advanced-stage carcinomas. Gain of 3q was identified as an early event, as it already occurred in 1 of 13 severe dysplasias.

An even clearer picture emerges in cervical carcinomas. While only 1 of the 13 severe dysplasias revealed a gain of the long arm of chromosome 3, 90% of stage I invasive carcinomas showed this particular chromosomal aberration. In advanced-stage carcinomas, the gain of chromosome arm 3q prevails, although with slightly reduced frequency (76%), and additional changes can be identified. The loss of sequences on chromosome arm 2q increases from 10% in stage I disease to 33% in advanced-stage diseases. Chromosome arm 5p was gained only once in stage I carcinomas; the incidence increases to 30% in advanced-stage disease, and was often identified as a whole-arm amplification. No alterations of 5p occurred in premalignant lesions. The subtractive karyogram allows the identification of chromosome arm 3q gain as an early event in cervical carcinogenesis. It seems obvious that, during the progression from stage I to stage IIb, III, and IV disease, additional chromosomal changes could be identified that were infrequently present in early carcinomas. These aberrations include the loss of a certain region on the long arm of chromosome 2, high-level copy number increases on 5p, as well as the acquisition of extra copies of chromosome arm 8q (data not shown). The subtractive karyograms also show that high-level chromosomal copy number changes (amplifications) occur late during tumor progression. High-level increases were rarely found in premalignant lesions or low-stage disease. Only 4.1% of all aberrations that were mapped in colorectal adenomas were amplifications, which increases to 20% in invasive carcinomas. Similarly, in preinvasive cervical dysplastic lesions no high-level copy number increases could be identified; in cervical carcinomas stage I, ampli-

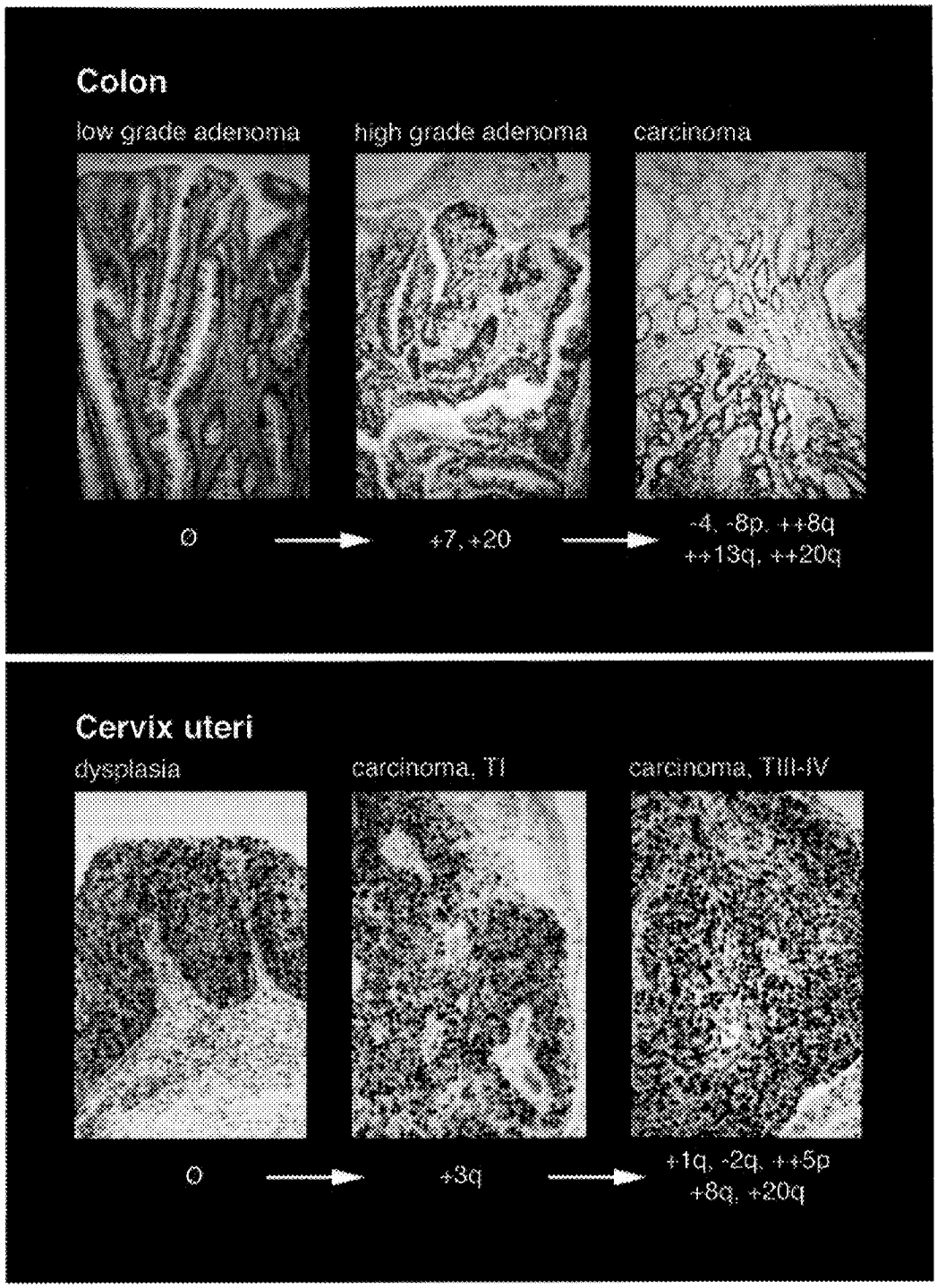

Figure 4. Stage-specific chromosomal aberrations in colorectal and cervical carcinogenesis. The results of subtractive karyograms are displayed in combination with the histomorphology in colorectal and cervical tumorigenesis.

fications are present at a frequency of 7% of all aberrations, which increases to 9% in the advanced-stage carcinomas. Examples of the subtractive karyograms are presented in Figure 3.

A summary is displayed together with tumor histology in Figure 4.

Interestingly, crude deviations from a normal, diploid DNA content could be observed at earlier stages than specific chromosomal copy number changes, both in colorectal and cervical tumors. Of note is that DNA ploidy changes precede the impairment of *TP53* function (Auer et al., 1994),

which occurs either via mutational inactivation (in colorectal tumors) or through functional inactivation by the E6 protein of human papilloma virus, which is detected in the majority of severe cervical dysplasia and invasive carcinomas. The lack of detectable chromosomal changes in early premalignant lesions, despite gross deviations of the nuclear DNA content, could have several reasons. First, chromosomal changes are random and therefore escape detection by means of CGH. Second, only a minority of the cells carries specific chromosomal changes, which again could result in normal CGH profiles but aneuploid DNA histograms. This question can obviously be addressed by using fluorescence in situ hybridization (FISH) with region-specific probes for recurrently involved chromosomes (such as chromosome arm 3q in cervical carcinomas) directly in tissue sections of premalignant and malignant disease. Interphase cytogenetics has a higher sensitivity than CGH, which detects copy number changes only if they are present in more than 50% of a cell population. If the second hypothesis holds true, it would be interesting to correlate the level of *TP53* expression with chromosomal aberrations in the same cells. Third, balanced chromosomal aberrations occurring are undetectable by CGH. The third possibility seems unlikely because balanced chromosomal aberrations would not affect the nuclear DNA content. In addition, the comparison of CGH results with conventional G-banding analyses of colorectal carcinomas has identified virtually a mirror image of the respective techniques (Bardi et al., 1995). This indicates that the majority of chromosomal aberrations do result in copy number changes (balanced chromosomal translocations would be detectable by banding, however, not by CGH). Therefore, reciprocal translocations with no effect on chromosomal copy numbers seem less common in colorectal and cervical tumors. This is in striking contrast to aberrations observed in hematological malignancies, in which proto-oncogene activation frequently occurs via juxtaposition of proto-oncogenes in the vicinity of activating regulatory sequences by means of cytogenetically detectable balanced translocations (Rowley, 1990; Rabbitts, 1994).

Phenotype/Genotype Correlation in Tumor Progression

Increased proliferative activity, genetic instability as reflected in crude DNA aneuploidy and the impairment of tumor suppressor gene function are common features of tumor progression (Hirano et al., 1994). We have thoroughly compared these parameters with copy number changes as detected by CGH. During both cervical and colorectal carcinogenesis increased proliferative activity was already observed in low-grade dysplastic lesions. The initial increase occurs prior to the manifestation of chromosomal aberrations and persists in invasive disease. Interestingly, the same observation holds true for crude cellular DNA content. Already in low-grade disease (classified as low-grade colorectal adenomas or mildly and moderately dysplastic cervical lesions), the cellular DNA content, as determined by image cytometry, revealed clear deviations from a diploid DNA content (Heselmeyer et al., 1996; Ried et al., 1996). Despite this deviation, specific chromosomal changes could not be identified, which indicates that tetra- and aneuploidization precedes the appearance of recurring chromosomal copy number changes in the majority of the cells. At the transition from preinvasive disease to invasive carcinomas, the number of copy number changes detected by CGH increases dramatically. This increase coincides with the loss of *TP53* function, which also occurs relatively late during carcinogenesis. Point mutations in the *TP53* gene have been shown to occur in more than 75% of colorectal carcinomas and are usually associated with loss of heterozygosity of that locus (Baker et al., 1989, 1990). *p21/WAF1* is a main downstream effector of *TP53* and mediates growth arrest by inhibiting the action of G1 cyclin-dependent kinases (El-Deiry et al., 1995). In colorectal carcinomas, an increase in immunoreactivity of the TP53 protein is accompanied by a weak or negative staining for *p21/WAF1* immunoreactivity. Such a pattern is compatible with the mutation inactivation of *TP53* and the subsequent lack of *TP53*-induced expression of *p21/WAF1* (El-Deiry et al., 1995). In cervical carcinomas, the immunophenotype is different. In contrast to many other human tumors, *TP53* mutations are only rarely detected in cervical cancers (Crook et al., 1992; Choo and Chong, 1993; Kessis et al., 1993; Busby-Earle et al., 1994). None of the 10 stage I invasive carcinomas included in our study revealed *TP53* mutations when analyzed using PCR-SSCP. We observed a predominantly weak or negative staining pattern for *TP53* expression. In strong contrast to the findings in colorectal tumors, immunocytochemically detectable levels of *p21/WAF1* are prevalent in about 50% of the cells in invasive carcinomas. Increased levels of *p21/WAF1* coincide with the detection of HPV genomes and occur at the transition of moderate to severe dysplasia. However, the persistence of HPV genomes provides an alternative mechanism for compromised *TP53* function via degradation of wild-type TP53 protein (Scheff-

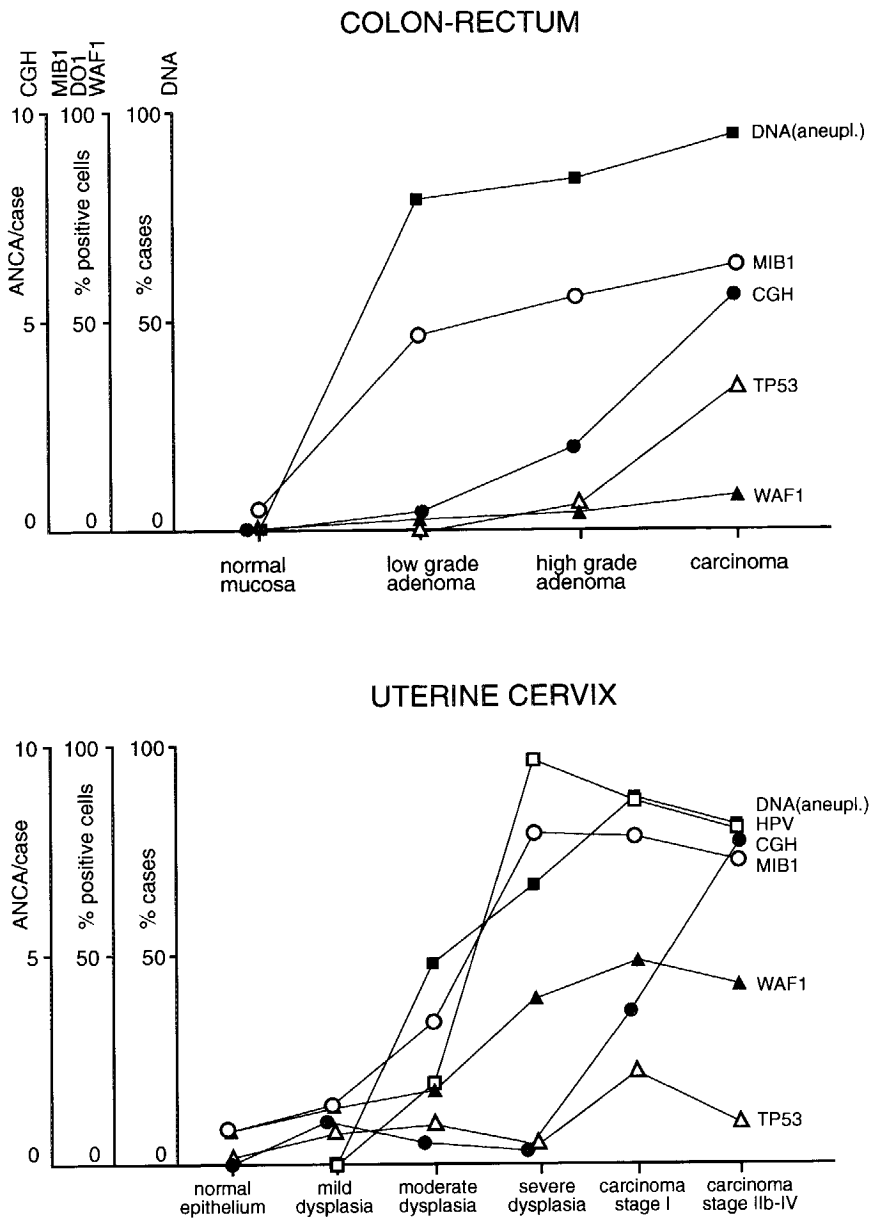

Figure 5. Correlation of nuclear DNA content, proliferative activity, expression levels of *TP53* and *p21/WAF1*, prevalence of HPV, and chromosomal aberrations in colorectal and cervical carcinogenesis. The graphs display the summary of all investigated parameters during colorectal and cervical carcinogenesis. Both in colorectal and cervical tumors, crude aneuploidy (DNA, solid squares) and increased proliferative activity (detected with the antibody MIB1, open circles) precede the fixation of specific chromosomal changes (CGH, solid circles). In colorectal tumors, *TP53* immunoreactivity increases notably at the adenoma-carcinoma sequence (*TP53*, open triangles), whereas *TP53* levels remain low in cervical carcinogenesis. *p21/WAF1* expression (WAF1, solid triangles) increases with tumor progression in cervical tumors, but is not induced during colorectal carcinogenesis. In both tumor types, the acquisition of recurring chromosomal aberrations occurs at the transition from premalignant lesions to invasive disease.

ner et al., 1990). Figure 5 presents the summary of the multivariable analysis in colorectal and cervical carcinogenesis.

We conclude that the reduction of wild-type *TP53* function at the transition from premalignant stages to invasive carcinomas is an important cellular event that coincides with the acquisition of recurring chromosomal aberrations in the majority of the tumor cells.

The data indicate that the histomorphologically defined steps of cellular dysplasia and the development of invasive carcinomas can be correlated with recurring chromosomal aberrations. The existence of tumor-specific, and even tumor stage-specific,

recurring chromosomal aberrations clearly indicates that such aberrations are mandatory events during carcinogenesis; this justifies their translational application to diagnostics (Ried, 1998). The characterization of the genetic makeup will allow one to assess biological variables as predictors for the progressive potential of tumor cells. In cervical carcinomas, for instance, it is clear that neither the existence of dysplastic cells nor the presence of HPV genomes are adequate indicators for the probability of a lesion to progress to invasive carcinoma. We hypothesize that the appearance of chromosomal aberrations, such as the invariably observed gain of chromosome arm 3q, are obligatory "second hits" for the irreversibility of the transformation progress. These specific chromosomal markers can be analyzed by interphase cytogenetics (Cremer et al., 1986), using clones for recurrently involved chromosomes and chromosomal bands and probes for specific oncogenes and tumor suppressor genes. One invaluable advantage of interphase cytogenetics is that the histo- and cytomorphological structures can be preserved. Therefore, genetic and chromosomal markers can be assessed simultaneously with morphology and even retrospectively in archived specimens. This should greatly facilitate the correlation of genetic markers with disease phenotype and clinical follow-up. It is conceivable that the application of such stage-specific markers to histological sections or cytological preparations will improve the diagnosis and staging of neoplastic disease, and in particular small tumors and noninvasive precursor lesions. The detection of a nonrandom pattern of genetic/chromosomal markers in clinical samples can evolve as a potent, individual predictor of the progressive potential of premalignant lesions and invasive carcinomas and will most likely be increasingly applied in diagnostic molecular pathology.

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